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BASIC SCIENCE ARTICLE

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Glucocorticoids in a Neonatal Hyperoxic Lung Injury Model: Pulmonary and Neurotoxic effects

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BACKGROUND: We aimed to compare the effect of dexamethasone (Dex), hydrocortisone (Hc), and methylprednisolone (Mpz) at equivalent doses on somatic growth, lung healing, and neurotoxicity in a hyperoxic rat model. We hypothesized that Mpz and Hc would be superior to Dex with less neurotoxicity by exerting similar therapeutic efficacy on the injured lung.
METHODS: Neonatal rats were randomized to control, bronchopulmonary dysplasia (BPD), Dex, Hc, and Mpz groups. All drugs were administered daily following day 15 over 7 days. Histopathological and immunohistochemical analyses of the lung and brain were performed on day 22.
RESULTS: All types had much the same impact on lung repair. Oxidative markers in the lung were similar in the steroid groups. While nuclear factor erythroid 2-related factor and heat-shock protein 70 dropped following steroid treatment, no difference was noted in other biochemical markers in the brain between the study groups. Apoptotic activity and neuron loss in the parietal cortex and hippocampus were noted utmost in Dex, but alike in other BPD groups.
CONCLUSIONS: Mpz does not appear to be superior to Dex or Hc in terms of pulmonary outcomes and oxidative damage in the brain, but safer than Dex regarding apoptotic neuron loss.

Pediatric Research (2022) 92:436–444; https://doi.org/10.1038/s41390-021-01777-z

IMPACT:

- This is the first study that compared the pulmonary efficacy and neurotoxic effects of Dex, Hc, and Mpz simultaneously in an established BPD model.
- This study adds to the literature on the importance of possible antioxidant and protective effects of glucocorticoid therapy in an oxidative stress-exposed brain.
- Mpz ended up with no more additional neuron loss or apoptosis risk by having interchangeable effects with others for the treatment of established BPD.
- Mpz and Hc seem safe as a rescue therapy in terms of adverse outcomes for established BPD in which lung and brain tissue is already impaired.

INTRODUCTION

There has been a considerable revolution in terms of diagnosis ever since the terminology of bronchopulmonary dysplasia (BPD) was brought up by Northway et al. for the first time in 1967.[1] Advanced survival of extremely preterm infants with the amelioration of less invasive perinatal care ended up with alterations in both pathogenesis and clinical course. "Old" BPD characterized mainly by fibrosis has given way to "New" BPD delineated as arrested development of alveoli with minimal fibrosis and airway injury.[2] While recent advances have led to notable progress in BPD prevention, the incidence has remained steady likely because of the higher survival rates of extremely preterm infants.[3,4] A plethora of research has been conducted to find out safe treatment strategies to cease and/or treat BPD. Systemic glucocorticoids and vitamin A have been proven to lower the incidence of BPD, by the alleviation of inflammation and oxidative damage.[3]

Oxygen radical disease of prematurity accounts for all prematurity-related morbidities.[5] Preterm babies, in particular, are susceptible to oxidative injury owing to the surpassing of oxidants on antioxidants that are yet functioning optimally due to immaturity.[5] Glucocorticoids possess well-delineated oxidative stress triggering effects in various tissues including the brain.[6] However, the mechanism of action within a hyperoxia-exposed brain and lung has yet to be elucidated.

Notwithstanding considerable discrepancies in the cumulative dose of the glucocorticoids given in various studies, early

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Received: 26 October 2019 Revised: 18 June 2021 Accepted: 12 September 2021
Published online: 1 November 2021

postnatal hydrocortisone (Hc) and late dexamethasone (Dex) have been demonstrated to diminish the risk of death/BPD without augmenting the risk of cerebral palsy.[4] However, current Cochrane meta-analyses claimed that the benefits of early or late glucocorticoid therapy might not prevail over adverse effects.[7,8] Hc has become the current topic of investigation in both clinical and experimental studies given the evidence of neurotoxic side effects induced mainly by high doses and early initiation of Dex.[9] Hc was suggested to diminish BPD without a conspicuous effect on survival, but with a higher incidence of intestinal perforation.[10] Merely, the results are still conflicting as to which glucocorticoid type has a maximal therapeutic impact on the lung with minimal neuronal damage, administered when and at which dose.

Methylprednisolone (Mpz) is the least investigated type in preterm babies[11–14] that might be promising with minimal neurological side effects with a shorter half-life and less glucocorticoid receptor stimulus. A similar respiratory outcome with less cystic periventricular leukomalacia with Mpz than Dex was inferred in a study.[11] A retrospective study drew attention to the effect of a brief Mpz course on diminishing oxygen dependency once given to infants with mild BPD at 36 gestational weeks.[12] Linafelter et al. designed a retrospective study with the usage of chronic prednisolone in infants with severe BPD, not newborn babies. Pulmonary outcomes recovered with prednisolone in short term, but with impaired linear growth.[14]

Unfavorable neurotoxic and growth restrictive impact of Dex has been well-designated in experimental work besides clinical studies.[15,16] The studies investigating Hc as an alternative therapy were promising in terms of lung healing without adverse neurodevelopmental outcomes along with weak evidence.[17–19] To the best of our knowledge, data are lacking regarding the comparison of Mpz with Hc, and Dex in equivalent doses in an established BPD model. We hypothesized Mpz and Hc would be superior to Dex with minimal neurotoxicity by the implementation of similar therapeutic strength on the injured lung. Therefore, we aimed to compare the pulmonary and neurological outcomes of Dex, Hc, and Mpz in an established hyperoxia-induced BPD rat model.

MATERIALS AND METHODS

Ethical approval

This experimental study was conducted in the Experimental Animals Laboratory in the Ankara Education and Research Hospital based on the Guide for Care and Use of Laboratory Animals suggested by the US Department of Health and Human Services. The Animal Research Ethical Committee in the same hospital approved the study.

Experimental design

Ten timed-pregnant Wistar albino rats were placed and cared for in separate individual cages with 12-h light–dark cycles in the animal laboratory. Free food and water were available ad libitum. Pregnant rats were allowed to give birth spontaneously.

The day of birth was named postnatal day 1 (PD 1). After delivery, pups were kept in room air on PD 1 and fed by the mother. Pups were weighed each day with a high-sensitivity (0.01 g) scale and weights were noted on PD 1, 15, and 22. All pups were delivered back to nursing dams in the cages. We set up a BPD rat model induced by postnatal hyperoxia.

Plexiglass containers were used to create hyperoxia. An oxygen sensor monitored the oxygen levels continuously with a Ceramatec (MAXO2) oxygen analyzer to maintain ≥85% O_2 saturation. Hyperoxic and normoxic chambers were alternately used every 12 h for Wistar albino rat dams to prevent lung injury. Litters were preserved as six pup-sized to keep standard nutrition. The experiment began on PD 2 and continued through PD 22. Humidity was maintained at 50%, and the environmental temperature was maintained at 24 °C.

On PD 2, eighty pups were pooled and randomly allocated to five groups based on the treatment they received while redistributing to nursing dams. Each group consisted of 16 pups. The control group received no drug therapy or intervention and was kept in room air. The BPD group was exposed to 85% O_2 and received an equivalent volume of saline. The glucocorticoid groups were exposed to 85% O_2 for 14 days and subsequently a 7-day drug treatment. Deaths of the pups were noted daily in all groups.

Dosing of corticosteroids

The dose of Dex was adjusted based on a previous study[16] and dosing of Hc and Mpz was calculated to provide an equivalent anti-inflammatory effect. Dex (Decort^R, Industry of Deva Drug), Hc (Hydrocort-Liyo^R, Industry of Kocak Farma), or Mpz (Prednol-L^R, Industry of Mustafa Nevzat) were administered to each of the three groups via intraperitoneal injection. All drugs were gradually tapered by 50% once every 2 days during a 7-day treatment between PD 15 and 21. Dex was commenced by a starting dose of 0.2 mg/kg/day (0.2, 0.1, 0.05, and 0.025 mg/kg/day, respectively) in the Dex group. The Hc group was administered 5 mg/kg Hc (5, 2.5, 1.25, and 0.6 mg/kg/day, respectively). Pups in the Mpz group received a similar gradually tapering dose from a starting dose of 1 mg/kg/day Mpz (1, 0.5, 0.25, and 0.125 mg/kg/day, respectively). The weights of the rats were determined from PD 1 to 22.

Harvesting of lung and brain tissues for histological and biochemical evaluation

Preparation of lung tissues. Anesthesia was performed using intraperitoneal ketamine (Ketalar^R, Industry of Pfizer) and xylazine (Basilazin^R, Industry of baVET) to rat pups on PD 22. After thoracotomy, cannulation was done through the trachea. Lungs were resected after perfusion of the heart with normal saline. Subsequently, lungs were fixed with the cannula by the implantation of normal saline at a constant inflation pressure of 5 cm H_2O. After suturing of the trachea, the right main bronchus was ligated with a surgical suture, removed, and saved for later biochemical analyses. The remaining left lungs were fixed in 10% neutral-buffered formalin for 24 h.

Preparation of brain tissues. Brain tissues, except the cerebellum, were removed quickly by transferring on ice. After removal, the brain weights were measured with a 0.01 g sensitive scale and recorded. All subsequent procedures were carried out at 4 °C in 30 s involving removal, weighing, and freezing. The right half of the brain was then immediately stored at −80 °C for subsequent biochemical analysis. The remaining left half of the brains were kept and fixed immediately in 10% neutral-buffered formalin for histopathologic and immunohistochemical examinations.

Histopathological examination of brain tissues. Brain tissues were processed and embedded in paraffin blocks. The blocks were cut into 4–5 mm sections with a microtome (Leica SM, Leica Biosystems Nussloch GmbH, Germany) at multiple levels and stained with hematoxylin–eosin and neuronal nucleic (NeuN, 1:400, Millipore, UK). All histopathologic analyses described were performed by an investigator with no prior knowledge of the treatment groups. For the detection of NeuN-positive cells in the brain, immunofluorescence was carried out. The hemispheric areas were determined at each cross-sectional level by one morphometrist with no prior knowledge of the treatment groups, using a computer-assisted image analyzer system consisting of a microscope (Olympus BH-2) equipped with a high-resolution video camera (JVC TK-890E camera; JVC, Yokohama, Japan). The images were processed with an IBM-compatible personal computer, high-resolution video monitor, and image analysis software. Briefly, the images were grabbed with the video camera at ×3.3 magnification; the hemispheric areas were viewed on the monitor and outlined by drawing.

Evaluation of brain samples (caspase-9, -8, and -3). Each sample was evaluated for all brain regions. The most affected regions of the developing brain were CA1, CA2/3, and dentate gyrus of the hippocampus and parietal cortex. The numbers of CA, CA2/3, dentate gyrus, and parietal cortex neurons were counted with the help of a 15,800 mm^2 counting frame viewed through a ×20 Nikon Lens on the monitor. The counting frame was placed randomly ten times on the image analyzer system monitor, the neuron numbers of CA1, CA2/3, and dentate gyrus regions of the hippocampus and parietal cortex were counted (UTHSCA Image Tool for Windows Version 3.0 software), and the average was taken. All counting and measurement procedures were performed blindly. For the determination of the percentage of apoptotic cells, ten regions were randomly selected from hippocampal regions and the parietal cortex for each

subject. Caspase-3 (1:100; [CPP32] Ab-4[rabbit PAP], 1 ml, Labvision [Thermo], RB-1197-P), caspase-8 (500 µl, Abcam, ab4052), and caspase-9 (LAP Ab-4) analyses were performed by staining with properly diluted primary antibodies for immunohistochemical study. One hundred cells were counted, and the number of caspase-8-, -9-, and -3-positive cells was noted for each region. Consequently, 1000 cells were counted for each subject, and the number of apoptotic cells was presented as a percentage.

Detection of neuron numbers. Neuron numbers were calculated in the hippocampus (CA1, CA2/3, and dentate gyrus) and parietal cortex, the sites with a plethora of corticosteroid receptors. Thinly sliced brain tissues were stained with caspase-3, -8, and -9. Under fluorescent microscopy with a 15,800 mm^2 counting frame viewed through a ×20 Nikon Lens, the number of caspase-3-, -8-, and -9-stained cells were counted in the previously defined most affected regions of the brain. Ten unique random areas from the hippocampal areas and parietal cortex were selected to determine the number of stained cells per mm^2. The blocks were stained with Neun.

Histopathological examination of the lungs. Lung tissues were processed and embedded in paraffin blocks. The blocks were cut into 4–5 mm sections with a microtome (Leica SM, Leica Biosystems Nussloch GmbH, Germany) at multiple levels. Hematoxylin–eosin technique was used to determine the lung histopathological grading, radial alveolar count (RAC), and mean linear intercept (MLI), as previously described.[20] The histopathological score was established with the following grades: grade 1, normal histology; grade 2, moderate leukocyte infiltration; grade 3, leukocyte infiltration, edema, and partial destruction; and grade 4, total tissue destruction.

The coefficients of the subepithelial and epithelial areas were obtained (which reflect cell infiltration and fibrosis) using morphometric evaluation of the lung tissue with the help of a computer program (Scion Image, 4.0.3) using a previously modified method.[19] For this, cross-sectional airway slices were selected that had been stained with Masson's trichrome, and the interior perimeters of the epithelium, the perimeters of the basal membrane, and the outer perimeters of the adventitia were plotted with the aid of a computer program (Scion Image, 4.0.3). The areas of these outlined slices were calculated to evaluate the subepithelial and epithelial area coefficients. ABC technique was used to detect lamellar body membrane protein expression with a previously defined immunohistochemical method.[21]

A computer-assisted image analyzer system including a microscope (Olympus BH-2) equipped with a high-resolution video camera (JVC TK-890E camera; JVC, Yokohama, Japan) was used by a blinded histopathologist who detected hemispheric areas. For handling the images, an IBM-compatible personal computer, high-resolution video monitor, and image analysis software were used. Briefly, the images were captured with the video camera at ×3.3 magnification; the hemispheric areas were viewed on the monitor and outlined by drawing. An investigator blind to the groups analyzed the histopathological examination.

Determination of oxidative protein damage, oxidative stress, and oxidative stress-derived DNA damage markers

Advanced oxidation protein products (AOPP) were measured by spectrophotometric analysis using a previously defined method.[22] Levels of tissue lipid hydroperoxide (LHP) were measured with commercially available kits (LPO Assay Kit, Item No. 705003, Cayman Chemical Company, Ann Arbor). 8-Hydroxydeguanosine (8-OHdG) was measured with the OXISLECT Oxidative DNA Damage ELISA Kit (Cell Biolabs, San Diego, CA). A rat CASP3 ELISA Kit (BioSource Europe S.A., Nivelles, Belgium) was used for the detection of tissue caspase-3 levels. Levels of nuclear factor erythroid 2-related factor 2 (Nrf2) were measured with the Nrf2 Transcription Factor Assay Kit, Item no. ER0666, Fine Test). Heat-shock protein 70 (HSP70) was measured with the HSP70 Elisa Kit (Cat. no: 201-11-0523, Sunred Biological Technology, China). Measurement of total thiol was carried out with Total Thiol Assay Kits (Rel Assay DC, Gaziantep, Turkey).

Determination of oxidative stress indexes, antioxidant enzymes, and myeloperoxidase

An autoanalyzer (Cobas Integra 800, Roche) with commercially available kits (Rel Assay Diagnostics Kit; Mega Medical, Gaziantep, Turkey) was utilized for the measurement of tissue total antioxidant status (TAS) and total oxidant status (TOS) by a method defined by Erel et al.[23] The formula of OSI = TOS/TAS was used for the calculation of the oxidative stress index (OSI). Glutathione (GSH) was measured with a previously described technique.[24] Myeloperoxidase (MPO) activity was determined using a method defined by Krawisz et al.[25]

Determination of inflammatory markers

Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) levels were measured in lung tissue using ELISA Kits (BioSource Europe S.A., Nivelles, Belgium) as per the manufacturer's instructions.

Statistics

SPSS (v21.0, IL) program was utilized for the analysis of the data. Kolmogorov–Smirnov test was used for testing for normal distribution of data. Definition of discrete factors was as proportions and continuous variables as the mean or median with their corresponding standard deviation or extreme values, respectively. Numerical variables were compared by analysis of variance and Bonferroni correction. Comparison of discrete variables was conducted by Fisher's exact test. A P value of <0.05 was described as a statistical significance in all two-tailed tests. The statistical power was analyzed. Taking a magnitude effect value of 0.60 and an alpha error of 0.05 and a beta error of 0.20 to obtain a statistical power of 80%, 16 animals were observed to be sufficient in each group.

RESULTS

Birth weights were statistically similar in the groups (5.42 ± 0.24, 5.51 ± 0.23, 5.48 ± 0.29, 5.44 ± 0.31, and 5.28 ± 0.23 g, respectively, in the control, BPD, Dex, Hc, and Mpz groups; $p > 0.05$). Body weight (BW) of all study groups on PD 22 was lower than the control group ($p < 0.05$). The results indicated statistically indistinguishable BW on PD 22 in the study groups ($p > 0.05$) (Fig. 1). All pups survived in the control group. While five pup deaths occurred in the BPD group, three pups of each study group passed during the study.

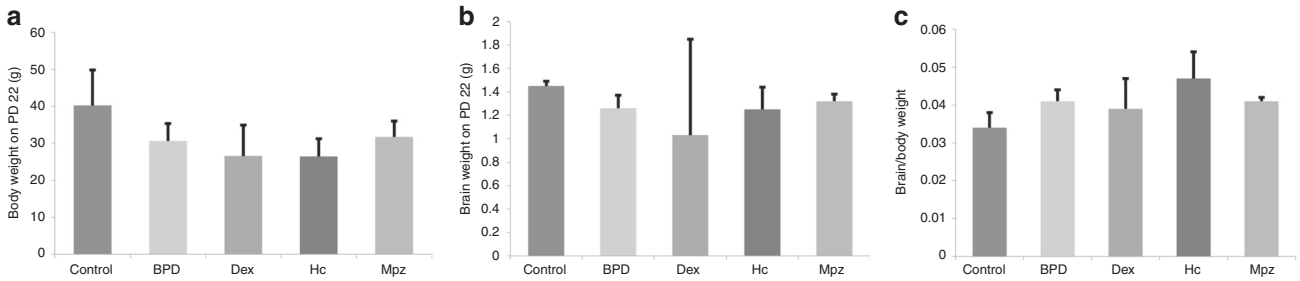

Fig. 1 Analysis of the body weight, brain weight, and brain weight/body weight. a Body weight (BW) on PD 22 was lower in all study groups than the control group (Control vs BPD $p = 0.007$, Control vs Dex $p = 0.001$, Control vs Hc $p = 0.001$, Control vs Mpz $p = 0.019$). The results indicated no significant difference in terms of BW between the four study groups ($p > 0.05$). **b** Brain weight was lower in the Dex group than in the control and other study groups ($p < 0.05$). No difference was present between Mpz, Hc, and BPD groups ($p > 0.05$). Of note, the brain weights of the control and Mpz pups were similar ($p = 0.429$). **c** The ratio of brain to body weight was higher in BPD, Hc, and Mpz groups than in the control group ($p < 0.05$). However, this ratio was notably similar in the control and Dex groups ($p = 0.231$). No significant difference was present between the study groups ($p < 0.05$). Data are presented as mean ± standard deviation.

Brain weights of BPD, Hc, and Dex pups were lower than the control group ($p = 0.041$, $p = 0.031$, and $p = 0.001$, respectively). However, pups in the control and Mpz groups had similar brain weight measurements ($p = 0.429$). Brain weights in the Dex group were statistically lower than the other study and control groups (Control vs Dex $p = 0.001$, Hc vs Dex $p = 0.001$, Mpz vs Dex $p = <0.001$). Pups who underwent Mpz, Hc, and O_2 treatment only (BPD) were noted to have statistically similar brain weights ($p > 0.05$) (Fig. 1).

BPD, Hc, and Mpz groups were found to have a higher brain to BW ratio than the control group (p values 0.012, 0.005, and 0.008, respectively). However, this ratio was notably alike in the control and Dex groups ($p = 0.231$). No significant difference was present between the study groups ($p < 0.05$) (Fig. 1).

Biochemical analysis of the lung tissue is shown in Table 1. While hyperoxia promoted a surge in TOS, OSI, AOPP, LHP, 8-OHdG, MPO, and caspase-3 release; TAS, GSH, and total thiol levels dropped. However, TOS, OSI, AOPP, LHP, 8-OHdG, and caspase-3 reduced following glucocorticoid administration; TAS, GSH, and total thiol levels went up without any difference between steroid groups. On the other hand, MPO was significantly lower in Dex than in other study groups.

The levels of TNF-α and IL-1β in the lung tissue were higher in the BPD group than in the control group. In response to glucocorticoid treatment, levels dropped significantly. However, the decrement of TNF-α was more prominent in the Dex group. IL-1β levels were similar in all study groups (Table 1).

In the histopathological examination of the lung, the severity of lung injury was categorized as grades 0–4 (Table 2). The control group had a normal alveolarization pattern. Impaired alveolarization characterized with alveolar septal thickening, simplification, enlargement, and reduction in the number and septation were detected in the BPD group (Fig. 2). Inflammatory cell infiltration was marked in the BPD group. RAC decreased and MLI increased with exposure to hyperoxia, as confirmed in the BPD group. A histological healing process following the treatment with glucocorticoids in the lung was displayed by an increment of RAC and decrement of MLI, but without returning to control values. Histopathological scores of the lung were the same in Dex, Hc, and Mpz groups. Lamellar body, which dropped following exposure to hypoxia, surged with glucocorticoid treatment. No difference was noted between Dex, Hc, or Mpz ($p > 0.05$).

Biochemical analysis of the brain tissue is shown in Table 3. AOPP, LHP, 8-OHdG, Nrf2, HSP70, and tissue caspase-3 surged in the brain following hyperoxia in the BPD group. Levels of these biomarkers did not significantly differ between BPD and glucocorticoid groups apart from HSP70, Nrf2, and caspase-3 ($p > 0.05$). HSP70 and Nrf2 levels diminished in response to glucocorticoids, but without any significant difference in corticosteroid groups. However, levels of tissue caspase-3 were higher in the Dex group than the others.

Histopathological examination and immunohistochemistry of the brain are demonstrated in Figs. 3 and 4. Caspase-3, -8, and -9 activity is noted to run up in both BPD and glucocorticoid groups.

Table 1. Biochemical analysis of lung tissues in the groups.

	Control	BPD	Dex	Hc	Mpz
TAS (mmol Trolox equivalent/g protein)	7.18 ± 0.94*	2.32 ± 0.39**	3.93 ± 0.57	3.76 ± 0.66	3.82 ± 0.89
TOS (μmol H_2O_2 equivalent/g protein)	6.34 ± 1.68*	39.42 ± 12.20**	21.17 ± 3.98	21.10 ± 2.23	20.93 ± 2.01
OSI (arbitrary units)	0.89 ± 0.23*	20.31 ± 4.82**	6.22 ± 1.12	6.52 ± 1.39	6.47 ± 1.24
GSH (nmol/g)	11.26 ± 3.42*	3.86 ± 2.25**	5.18 ± 1.89	5.30 ± 1.80	5.12 ± 1.56
AOPP (ng/mg protein)	5.07 ± 2.47*	17.54 ± 2.72**	11.31 ± 5.46	7.73 ± 3.61	7.40 ± 2.82
Lipid hydroperoxide (nmol/l)	0.572 ± 0.0847*	1.29 ± 0.417**	0.86 ± 0.12	0.656 ± 0.151	0.768 ± 0.165
8-OHdG (ng/ml)	1.605 ± 0.363*	6.86 ± 1.72**	3.34 ± 1.79	3.73 ± 2.66	3.48 ± 1.85
Total thiol (μmol/L)	0.744 ± 0.165*	0.288 ± 0.130**	0.423 ± 0.065	0.428 ± 0.046	0.412 ± 0.053
MPO (ng/mg protein)	32.29 ± 16.12*	157.16 ± 25.21**	82.31 ± 15.9$^{\alpha}$	91.3 ± 12.15	88.97 ± 10.95
TNF-α (pg/mg protein)	61.5 ± 7.5*	188.6 ± 18.6**	117.3 ± 12.3$^{\alpha}$	141.25 ± 29.3	142.2 ± 9.8
IL-1β (pg/mg protein)	29.7 ± 6.5*	67.3 ± 7.6**	44.6 ± 10.6	47.3 ± 7.8	42.5 ± 5.7
Caspase-3 (ng/g protein)	9.2 ± 3.4*	43.8 ± 7.4**	21.1 ± 5.3	22.4 ± 6.1	21.3 ± 4.9

All variables are defined as mean ± standard deviation.
AOPP advanced oxidation protein products, GSH glutathione, IL-1β interleukin-1β, 8-OHdG 8-hydroxydeoxyguanosine, OSI oxidative stress index, TAS total antioxidant status, TNF-α tumor necrosis factor-α, TOS total oxidant status, MPO myeloperoxidase.
*$p < 0.001$: comparison of control with all BPD groups.
**$p < 0.001$: comparison of BPD with glucocorticoid groups.
$^{\alpha}p < 0.001$: comparison of Dex with Hc and Mpz groups.

Table 2. Histopathological examination of the lung tissue.

	Control	BPD	Dex	Hc	Mpz
Histopathological score[a]	1(0)*	3(2)	2(1)	2(1)	2(1)
RAC[b]	12.5 ± 2.68*	5.2 ± 2.1	8.7 ± 2.5	9.1 ± 2.2	8.9 ± 2.5
MLI (μm)[b]	47.34 ± 3.67*	69.53 ± 4.87	56.53 ± 4.87	57.34 ± 5.68	55.89 ± 4.92
Lamellar body (Type 2 cells/mm^3)[b]	228.5 ± 10.5*	76.6 ± 22.4	118.8 ± 19.4	116.2 ± 21.3	110.7 ± 18.4

RAC radial alveolar count, *MLI* mean linear intercept.
*$p < 0.05$: comparison of control with BPD, Dex, Hc and Mpz groups.
[a]Median (interquartile range).
[b]Mean ± standard deviation.

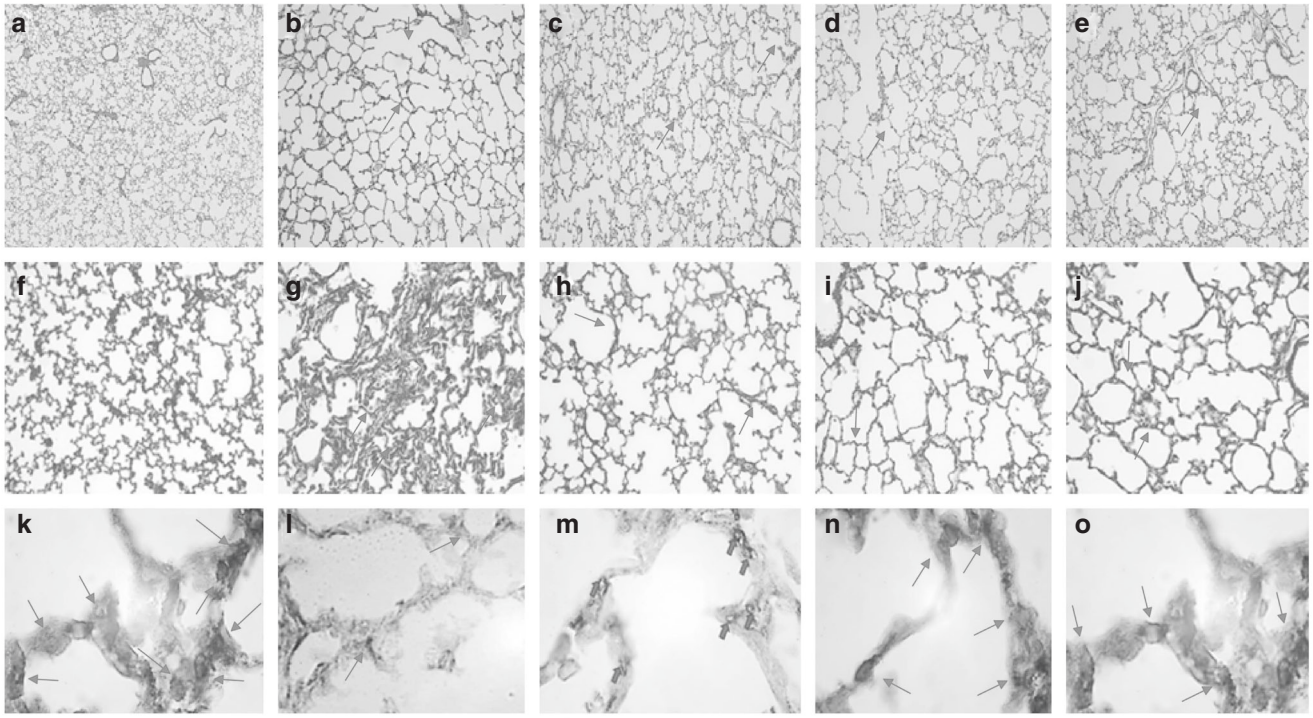

Fig. 2 Images demonstrating histopathological changes in the lung of rat pups in each group. Hematoxylin–eosin stain (**a**–**e**, ×100), Masson's trichrome stain (**f**–**j**, ×100), and immunohistochemistry for lamellar body membrane protein (**k**–**o**, ×400). Histopathological scores were the same in Dex (**c**), Hc (**d**), and Mpz (**e**). RAC dropped, and MLI surged in BPD (**g**). The reverse happened with the surge of RAC and the decrement of MLI following the administration of Dex, Hc, and Mpz (**h**–**j**). Lamellar body, which was reduced after exposure to hyperoxia, was triggered with glucocorticoid therapy. No difference was remarked between Dex, Hc or Mpz groups (**m**–**o**).

The Dex group was noted to have the maximum percentage of cells positive for caspase-3, -8, and -9 ($p < 0.05$). However, apoptotic activity was similar between BPD, Mpz, and Hc groups. The rates of apoptosis had significant similarities in various regions of the brain (Fig. 3).

Hyperoxia-induced neuron loss in all BPD groups (Fig. 5). The number of Neun-stained cells significantly reduced in Dex. Peak neuron loss was detected in the Dex group.

However, no more additional neuron loss was stated in Mpz or Hc groups over the BPD group.

DISCUSSION

In this study, we compared the somatic growth, pulmonary, and neurological outcomes of Dex, Hc, and Mpz in an experimental hyperoxia-induced established BPD model. Histopathological findings and biochemical markers in the lung, with a couple of exceptions, were similar in all glucocorticoid groups. On the other hand, TNF-α and MPO were significantly lower in Dex, reflecting a potent anti-inflammatory effect in the lung at high doses with longer therapy. Supporting already existing data, all glucocorticoid types led to a restriction in PWG and brain weight. While the brain weight of Dex pups was lower than the ones in BPD, Mpz, and Hc, no significant difference whatsoever was noted between Mpz, Hc, and BPD. The ratio of brain weight to BW was much the same in the control and Dex groups. BPD, Hc, and Mpz groups were noted to have a higher brain to BW ratio than the control group likely because of more pronounced adverse effects on postnatal weight gain than brain weight. However, all study groups were noted to have similar ratios. Hsp70 and Nrf2 dropped in response to glucocorticoid treatment in keeping with the antioxidant effect. Neuron loss in the parietal cortex and subareas of the hippocampus was higher with Dex, but much the same in Hc, Mpz, and BPD groups. Besides, caspase-3, -8, and -9 activity were at a higher percentage in Dex than in Hc, Mpz, and BPD. However, the percentage of caspase-positive cells was almost identical in Mpz, Hc, and BPD groups. Also, tissue caspase-3 levels were significantly higher with Dex than Hc and Mpz. Therefore, we are adding new data on the pre-existing literature that no more additional neuron loss or apoptosis risk occurred following Mpz with an interchangeable effect with others when used for the treatment of established BPD.

Glucocorticoids are well-known with favorable effects on pulmonary outcomes in preterm babies with BPD. The exact mechanism of action on the injured lung tissue remains unknown along with evidence showing the amelioration on gas exchange, surfactant production, antioxidant and anti-inflammatory effects.[26] However, glucocorticoids were revealed to disrupt alveolarization, diminish surface area and trigger emphysematous changes once given during the early postnatal period in a rat study.[27] From this point of view, the pulmonary benefits of steroid treatment seem to be associated with what type of lung tissue the glucocorticoids are given to.

Despite the unquestionable advantages of Dex on impaired lung structure, investigations focused on alternative types that have minimal adverse effects.[28] Dex and Hc were compared in terms of the effect on the alveolar growth of the lung without any previous intervention in a rat model.[27] Both Dex and Hc, despite less detrimental effects, altered lung morphology even in tiny doses given on PD 4–14, which corresponds to the alveolarization phase. On the other hand, the mechanism of action is amended totally when applied to an injured lung. For instance, in a rat model of hyperoxic BPD lung, both drugs impaired proper lung growth in rats with BPD, but with less deleterious findings with Hc compared to Dex.[29] Both drugs ameliorated the lung histopathological findings in our study as well. Nevertheless, the impact of Dex and Hc on the lung was much similar in terms of the histopathological changes on the contrary of the study, which was

Table 3. Biochemical analysis of brain tissue in the groups.

	Control	BPD	Dex	Hc	Mpz
AOPP (ng/mg protein)	2.36 ± 0.96*	7.75 ± 2.45	7.43 ± 1.67	7.14 ± 1.45	7.33 ± 1.21
Lipid hydroperoxide (nmol/l)	0.743 ± 0.052*	1.96 ± 0.684	1.83 ± 0.54	1.71 ± 0.491	1.69 ± 0.165
8-OHdG (ng/ml)	2.11 ± 0.52*	8.78 ± 2.34	9.12 ± 3.06	8.98 ± 2.89	9.25 ± 3.14
Nrf2 (pg/ml)	120.27 ± 32.70*	193.55 ± 24.12**	166.67 ± 21.75	171.43 ± 52.52	162.43 ± 36.10
HSP70 (ng/ml)	352.48 ± 22.11*	499.15 ± 32.29**	449.45 ± 19.16$^{\alpha}$	439.74 ± 9.43	433.63 ± 19.14
Tissue caspase-3 (ng/g protein)	6.4 ± 2.6*	52.1 ± 12.8**	65.1 ± 5.8$^{\alpha}$	54.3 ± 8.4	53.5 ± 9.1

All variables are defined as mean ± standard deviation.
*AOP*P advanced oxidation protein products, *Nrf2* nuclear factor erythroid 2-related factor 2, Hsp70 heat-shock proteins, 8-OHdG 8-Hydroxydeoxyguanosine. While hyperoxia-induced increases in AOPP and lipid hydroperoxide, 8-OHdG reduced in response to hyperoxia. However, either glucocorticoid administration decreased Nrf2 and HSP70 that is a remarkable finding for the possible antioxidant action in hyperoxia-injured brain injury.
*$p < 0.001$: comparison of control with BPD, Dex, Hc, and Mpz groups.
**$p < 0.001$: comparison of BPD with glucocorticoid groups.
$^{\alpha}p < 0.001$: comparison of Dex with Hc and Mpz groups.

resembling ours in terms of the hyperoxia model and timing of the intervention.

The impact of Mpz on the newborn lung is yet to be clarified. Mpz was hypothesized not only as effective as Dex but also safer in terms of adverse effects with a different genomic and non-genomic potency, namely receptor affinity and molecular structure in newborn infants. In an experimental study, Mpz altered cell proliferation and lung growth without leading to growth restriction in little doses when administered to fetal rabbits at the canalicular stage.[30] In a clinical study, a tapering protocol of Mpz on mean PD 16 was indicated as potent as Dex for reducing BPD incidence with less growth restriction and cystic periventricular leukomalacia.[11] Also, Bhandari et al. showed the benefit of oral prednisolone for weaning supplemental oxygen in mild BPD, but not effective enough in severe cases.[12] However, Linafelter et al. showed the recovery of pulmonary outcomes with chronic prednisolone treatment in infants with severe BPD.[14] Our study put forth equivalent potency of Mpz with Dex and Hc on lung healing as supported by histopathological scores and biochemical analyses.

Unfavorable neurodevelopmental outcomes were reported in numerous clinical and experimental studies.[15–19] One potential mechanism might be the trigger of oxidative stress by augmentation of the release of reactive oxygen species and eventual apoptosis in the hippocampus and cortex.[6,31] In a rat study, Dex promoted the expression of Hsp70 once coadministered with an antioxidant, Vitamin C.[32] Also, Dex was noted to alter the transcription of Nrf2, a basic leucine zipper protein that is responsible for the coordination of the expression of antioxidant proteins that defend against oxidative injury.[33] It has a pivotal protective role against oxidative damage in the brain. In our study, the expression of oxidative markers as AOPP, LHP, 8-OHdG, Nrf2, and Hsp70 surpassed in the hippocampus and parietal cortex following exposure to hyperoxia. Hsp70 expression bumps up in the brain in response to oxidative injury regarding the protective activity in nervous system injury. Hsp70 has a key role as a chaperone and keeps neurons apart from protein aggregation and toxicity, and eventually apoptosis. Besides these oxidation markers, caspases, a group of intracellular inactive pro-enzymes, were used as biomarkers for apoptosis. Caspase-8 is responsible for leading off the pathway, then caspase-9 activates and sustains segregation, and both activates caspase-3, which disintegrates critical cellular proteins or other caspases. The oxidative injury was emphasized to be correlated with neurodevelopmental disorders even beyond the newborn period.[34] Moreover, a close association between lung and brain injury induced by hyperoxia has been highlighted in recent papers.[35,36] Therapies targeting hyperoxic lung injury were hypothesized to co-alleviate hyperoxic brain injury based on this association. Inhaled nitric oxide and intratracheal transplantation of mesenchymal stem cells reverted not only lung damage to some extent but also brain injury in rats who underwent hyperoxia.[35,36] Even if the exact mechanisms by which these lung targeted therapies provide neuroprotection remains unclear, it was hypothesized to be mediated by anti-inflammatory, antioxidative, and antiapoptotic effects. In this study, hyperoxia ended up with simultaneous lung and brain damage potentially by previously speculated mechanisms. No further immunohistochemical brain injury was noted following Mpz or Hc treatment in addition to the hyperoxia injury seen in the BPD group. However, Nrf2 and HSP70 levels dropped in all steroid groups. As a novel finding, glucocorticoids were noticed to possess neuro and pulmonary protection by antioxidative mechanisms in damaged lungs and the brain. So, if this information could be supported in human beings, Mpz and/or Hc might become promising therapies in very preterm infants with severe BPD in long term.

The restriction in brain weight gain was more remarkable in Dex than in Hc as shown in previous experimental studies.[15–17] Dex stimulated apoptosis in the hippocampus and other regions of the developing brain in rat studies.[15–17] Besides, distorted hippocampal synaptic function and memory formation with Dex was long-lasting until later life.[37] In simultaneous human studies, Dex given in the early postnatal period was associated with low brain volume at adolescent age.[38] However, Hc did not have a pronounced detrimental impact on PWG, brain growth, or programmed neuron loss with the lowest glucocorticoid receptor affinity, shorter half-life, and similarity to endogenous cortisone in the recent work.[17,19] Merely, Hc was as neurotoxic as Dex in a chicken study.[39] Thus, the exact mechanism of action of Hc on the brain has yet to be elucidated. However, glucocorticoid was given to healthy animals in these experimental studies. As opposed to those, the Hc group showed lower caspase activity as compared to Dex, but similar to the BPD group. This finding emphasized no more extra neurotoxicity risk with Hc in the already maldeveloped brain triggered by oxidative injury.

Mpz ended up with no significant difference in terms of brain and BW compared to the BPD group in our study. In an experimental study, Kilic et al. compared the impact of Mpz and Dex on rat growth and neurological outcomes once administered on PD 3–5.[40] Growth restriction was less, and total neurological scores were lower in the Mpz group. Whilst a lower neurological score was reported in Mpz than in control on PD 7 and 14, there was no difference on PD 21. In another experimental study, Duksal et al. revealed a higher apoptotic index in the hippocampus and CA subregions with high-dose Dex than Mpz, but minimal with low-dose Dex once implemented on PD 3–5.[18] However, the ratio of brain to BW was more than high-dose Dex, but not different from low-dose Dex. In our study, caspase activity was lower in Mpz than in Dex, similar to Hc. However, we administered much later

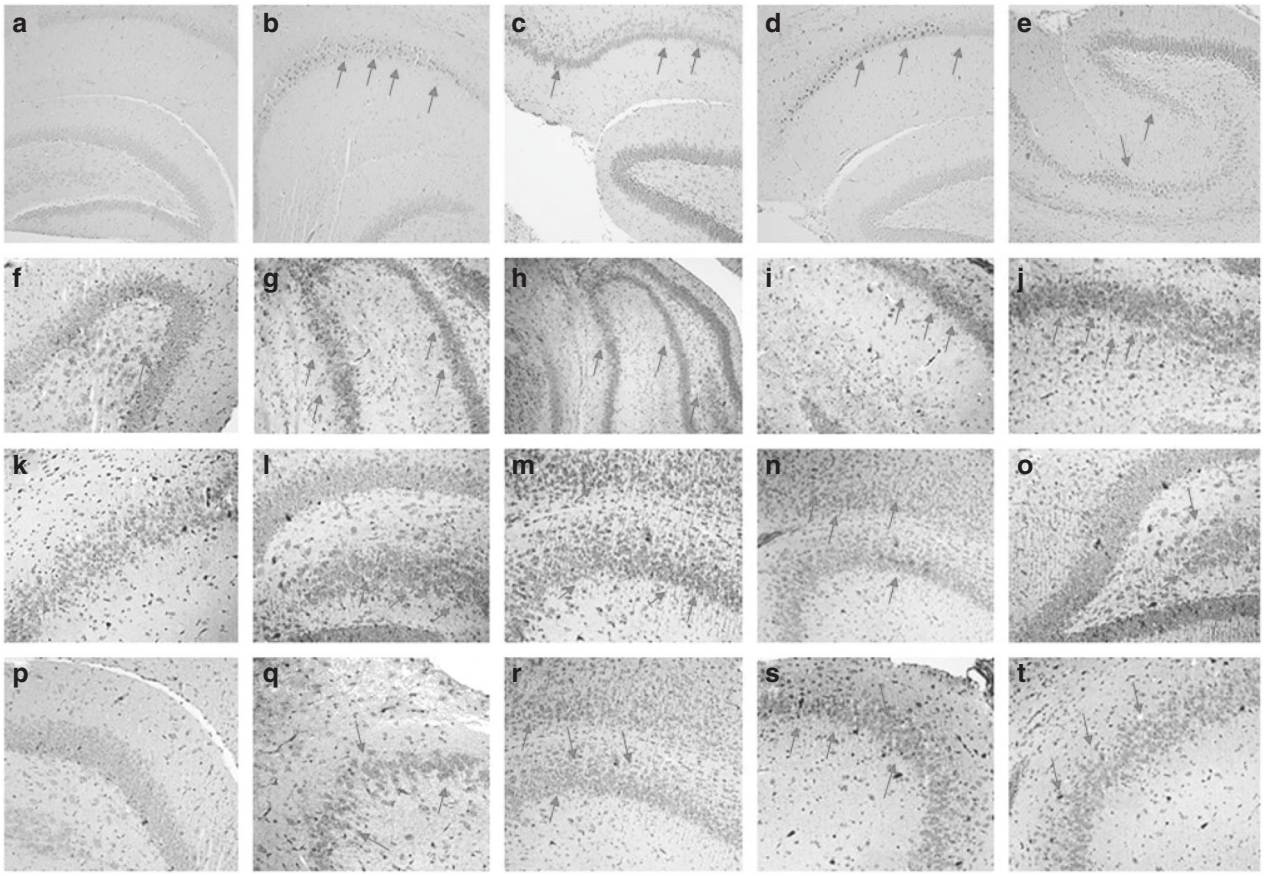

Fig. 3 Hematoxylin–eosin stain (×100) and immunohistochemical analysis of the groups in parietal cortex and hippocampus (×200). The control group (**a**) had normal histological findings of the hippocampus. BPD group (**b**) was noted to have the highest number of neuronal degeneration (red arrows). While Dex group (**c**) showed remarkable neuronal degeneration, the density of neurodegeneration was similar and less in Hc (**d**) and Mpz (**e**) groups. Caspase-9, -8, and -3 immunoreactivity (neuronal apoptosis indicated by red arrows) was minimal in the control group (**f**, **k**, and **p**). Caspase-9, -8, and -3 neurons were significantly higher in the BPD group than those in the control group (**g**, **l**, and **q**). Dex pups had more Caspase-9, -8, and -3 positive neurons than those in the Hc and Mpz groups (**h**, **m**, and **r**).

treatment beyond the newborn period as opposed to these studies in which the timing of the intervention was earlier. Moreover, Dex was implemented with two doses in the other study unlike ours. The disparities of study setting in terms of timing of intervention, doses, and hyperoxia model might have led to diverse conclusions from these experimental works. By any means, Mpz seems more reassuring than high-dose Dex, but not the lower dose, in terms of neurological outcomes regardless of the timing of intervention.

To the best of our knowledge, this is the first study that compared the pulmonary efficacy and neurotoxic effects of Dex, Hc, and Mpz in an established BPD model. As far as we know, we put forward potential antioxidant effects of glucocorticoids in a hyperoxia-exposed brain for the first time. We set up the study as Hc and Mpz being given at equivalent doses with Dex to document the efficacy and side effects comprehensively. We preferred prolonged 14-day hyperoxia-induced BPD to establish a thorough oxidative injury model. Several papers used this hyperoxia model. Kim et al. seized upon a similar hyperoxia model in multiple experimental researches.[36,37] Unlike the up-to-date tendency to commence on earlier and short-lasting steroids mainly aiming to stop evolving BPD, the purpose of this study was to interpret the efficacy of chronic steroid therapy in extremely preterm infants with established severe BPD. However, despite the prolonged and higher-dose Mpz and Hc course with tapering, less apoptosis and no significant brain weight loss indicates safety in terms of neurodevelopmental outcome. Besides, this study has some limitations. Various experimental hyperoxia-induced established BPD models were identified in the literature,[41] but it has yet to be clarified which model is the ideal one. Duration and level of hyperoxia exposure were reported as a minimum in a bunch of studies. The fact remains that the BPD model built up by hyperoxia might not be reflecting the pathology of "new" BPD, which is the current presentation nowadays. Notwithstanding equivalent doses, the metabolism of the glucocorticoids might diverge between rats and humans. Again, one of the noteworthy limitations of this study is the postneonatal timing of drug intervention in rats. Therefore, it is quite a late period, not neonatal, from which it is hard to directly extrapolate to human infants with established BPD. Previous studies have reported wide variability regarding the dosing and timing of the drugs. However, the timing of the intervention has been acknowledged to completely amend the degree of neurotoxicity due to the vulnerability of the developing brain in early life. So, late administration beyond the neonatal period might have avoided the emergence of adverse outcomes with Mpz or Hc.

In conclusion, Mpz and Hc seem to be reassuring as a rescue therapy with no extra risk in terms of unfavorable neurological outcomes for already developed severe established BPD. Dex ended up with a negative impact on somatic growth and led to apoptosis. All drugs look like having similar therapeutic consequences on BPD lung, apart from the pronounced anti-inflammatory potency of Dex. Further experimental and randomized human clinical trials are urgently required to set forth clear-cut therapeutic and adverse

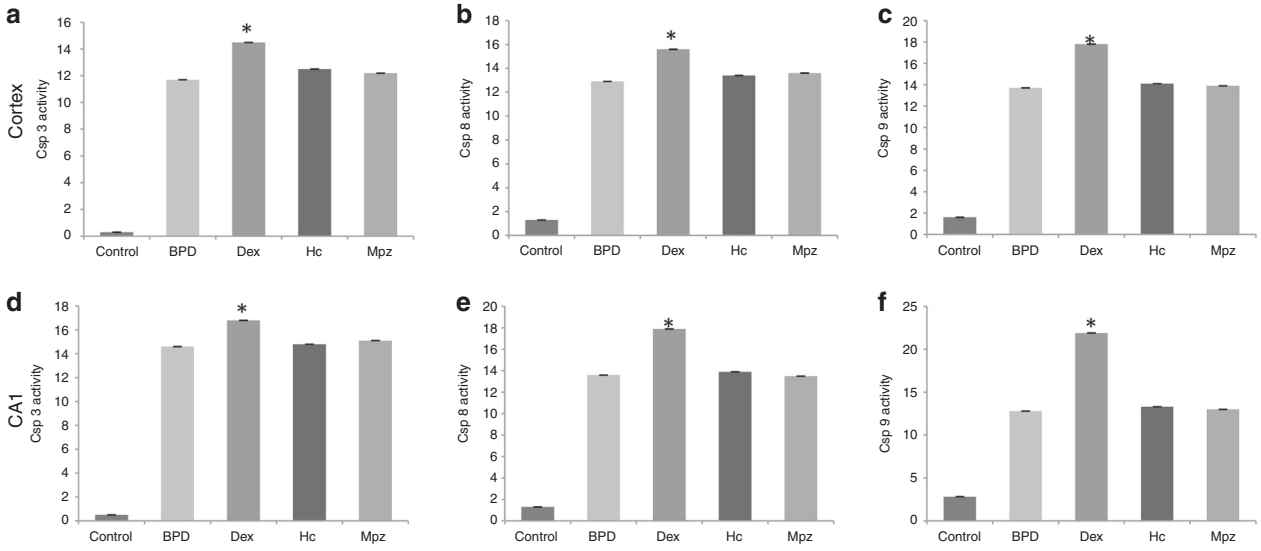

Fig. 4 Immunohistochemistry for caspase-9, -8, and -3 staining of the study groups for parietal cortex, CA1, CA2, and CA3 and dentate gyrus. The most pronounced apoptotic activity was detected in the Dex group in all assessed regions of the brain. Data are presented as a percentage. *$P < 0.01$ vs other groups.

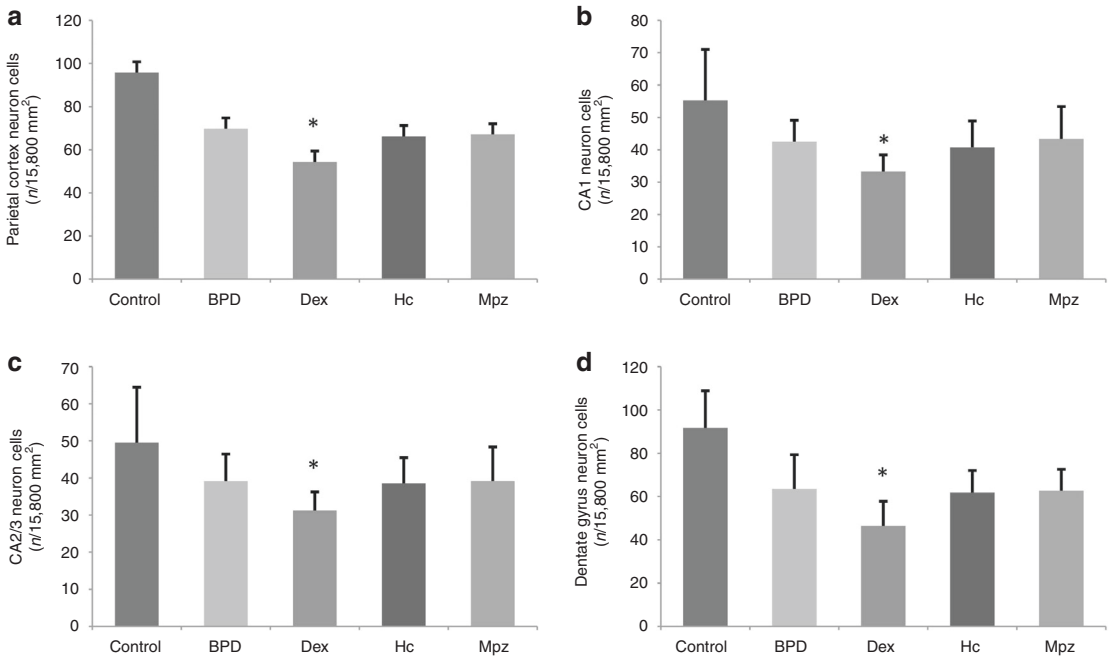

Fig. 5 Neuron numbers in the parietal cortex and subregions of the hippocampus. Hyperoxia induced neuron loss in all BPD groups. Peak neuron loss was detected in Dex group in all regions of the brain studied. However, no more additional neuron loss was stated in Mpz or Hc groups over the BPD group. Data are presented as mean ± standard deviation. *$P < 0.01$ vs other groups.

outcomes of Mpz and Hc. Despite decent evidence regarding the pulmonary benefits, a clear recommendation on the usage of glucocorticoids could not have been made for BPD prevention or treatment given the well-known neurotoxicity.

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AUTHOR CONTRIBUTIONS

B.O.B. and C.T. designed the research; B.O.B., C.T., M.B., U.C. and E.C. conducted the research and performed the experiments. B.O.B., C.T., and U.C. analyzed the data and made statistical analyses. I.K. performed biochemical analyzes and T.T.T. performed pathological examinations. B.O.B., C.T. and U.C. wrote the paper with revision. B.O.B. and C.T. had primary responsibility for the final content.

FUNDING INFORMATION

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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